METHOD FOR CONDUCTING THE SYNTHESIS OF NUCLEIC ACID MOLECULES

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Inventor: BERNAUER ANNETTE (DE)
Applicant: BERNAUER ANNETTE (DE)
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Abstract of **WO9947536**

The invention relates to a method for conducting the synthesis of nucleic acid molecules. The invention especially relates to a method which is carried out in a recursive manner. The nucleic acid constituents are preferably of a synthetic or semisynthetic origin. According to the inventive method, an additional nucleic acid molecule is attached to and/or coupled with a prepared nucleic acid molecule. The end of the prepared nucleic acid molecule is masked if no additional nucleic acid molecule is attached to or coupled with the same. The additional nucleic acid molecule is split at a predetermined point, resulting in an end to or with which an additional nucleic acid molecule can be attached and/or coupled. The aforementioned method steps can be repeated as often as required until the desired product is synthesized. The invention also relates to a kit for carrying out the inventive method.

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(71)(72) Anmelder und Erfinder: BERNAUER, Annette [DE/DE]; Weberstrasse 38, D–79249 Merzhausen (DE).

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(54) Title: METHOD FOR CONDUCTING THE SYNTHESIS OF NUCLEIC ACID MOLECULES

(54) Bezeichnung: VERFAHREN ZUR SYNTHESE VON NUCLEINSÄUREMOLEKÜLEN

(57) Abstract

The invention relates to a method for conducting the synthesis of nucleic acid molecules. The invention especially relates to a method which is carried out in a recursive manner. The nucleic acid constituents are preferably of a synthetic or semisynthetic origin. According to the inventive method, an additional nucleic acid molecule is attached to and/or coupled with a prepared nucleic acid molecule. The end of the prepared nucleic acid molecule is masked if no additional nucleic acid molecule is attached to or coupled with the same. The additional nucleic acid molecule is split at a predetermined point, resulting in an end to or with which an additional nucleic acid molecule can be attached and/or coupled. The aforementioned method steps can be repeated as often as required until the desired product is synthesized. The invention also relates to a kit for carrying out the inventive method.

(57) Zusammenfassung

Die Erfindung betrifft ein Verfahren zur Synthese von Nucleinsäuremolekülen. Insbesondere betrifft die Erfindung ein derartiges Verfahren, das rekursiv durchgeführt wird. Die Nucleinsäurekomponenten sind vorzugsweise synthetischen oder semisynthetischen Ursprungs. Das Prinzip des erfindungsgemässen Verfahrens beruht darauf, dass an bzw. mit einem bereitgestellten Nucleinsäuremolekül ein weiteres Nucleinsäuremolekül angelagert und/oder verknüpft wird, das Ende des bereitgestellten Nucleinsäuremoleküls maskiert wird, falls an dieses bzw. mit diesem kein weiteres Nucleinsäuremolekül angelagert und/oder verknüpft wurde, das weitere Nucleinsäuremolekül an einer vorbestimmten Stelle gespalten wird, wobei vorzugsweise wiederum ein Ende entsteht, an das bzw. mit dem ein weiteres Nucleinsäuremolekül angelagert und/oder verknüpft werden kann, und die vorgenannten Verfahrensschritte gegebenenfalls so oft wiederholt werden, bis das gewünschte Produkt synthetisiert ist. Die Erfindung betrifft ferner einen Kit zur Durchführung des erfindungsgemässen Verfahrens.

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Method to the synthesis of Nucleinsäuremolekülen the invention betrifft a method to the synthesis of < RTI ID=1.1> Nucleinsäuremolekülen. In particular betrifft< /RTI> the invention a such method, which < recursively; RTI ID=1.2> durchgeführt< /RTI> becomes. The Nucleinsäurekomponenten is preferably synthetic or semisynthetischen origin. The principle < RTI ID=1.3> erfindungsgemässen< /RTI> Method been based < RTI ID=1.4> whereupon, < /RTI> < RTI ID=1.5> that an< /RTI> and/or. with a made available Nucleinsäuremolekül a further Nucleinsäuremolekül angelagert and/or < RTI ID=1.6> verknüpft< /RTI> , the end of the made available is < RTI ID=1.7> Nucleinsäure < /RTI> molecule masked becomes, if to this and/or. with this no further Nucleinsäuremolekül was angelagert or linked and, which is split further Nucleinsäuremolekül in a pre-determined place, whereby preferably again an end develops, to that and/or. with a further Nucleinsäuremolekül angelagert and or < RTI ID=1.8> verknüpft< /RTI> will can, and which are so often repeated if necessary aforementioned process steps, until the desired product is synthesized. The invention < RTI ID=1.9> betrifft< /RTI> furthermore a kit to the lead-through < RTI ID=1.10> Verfahrens.< according to invention; /RTI>

Rekombinante techniques for the manipulation of Nucleinsäuren have many scientific disciplines in the last twenty years, in addition, lent an enormous buoyancy to the pharmaceutical industry as well as the medicinal research. < RTI ID=1.11> In vielen< /RTI> Ranges of application is desirable it, a Nucleinsäuremolekül with exactly defined sequence on < RTI ID=1.12> möglichst< /RTI> simple way with only small Zeit-und cost < RTI ID=1.13> bereitzustellen.< /RTI> < RTI ID=1.14> gegenwärtig< /RTI> at the furthest common method for the supply of such Nucleinsäuremoleküle contain < RTI ID=1.15> Clonierung< /RTI> of
DNA for example from cDNA Genbanken, if necessary coupled with following
Sequenzierung of the insulated < RTI ID=1.16> cDNA.< /RTI> On the other hand can DNA with desired sequence synthetic, for example < RTI ID=1.17> über< /RTI> < RTI ID=1.18> konventionelle< /RTI> Phoshoamidit method, < RTI ID=1.19> hergestellt< /RTI> become.

< RTI ID=1.20> Obliche< /RTI> Methods for supply desired doppelsträngiger Nucleinsäuremoleküle are < in the following by the example of the supply of; RTI ID=1.21> DNA Molekulen< /RTI> < RTI ID=1.22> erläutert.< /RTI> < RTI ID=1.23> Interessierende< /RTI> DNA molecules < RTI ID=1.24> müssen< /RTI> for example by one < RTI ID=1.25> cDNA oder< /RTI> < RTI ID=1.26> Positionierungsclonierung< /RTI> insulated and into suitable Vectors to be cloniert. The Vermehrung of the resulting vectors and thus the interesting
DNA molecules < RTI ID=1.27> taken place " in< /RTI> vivo ". In addition the vectors must into suitable Wirtszelien, for example
Bacteria or yeast, to be brought in. To the further manipulation of the DNA, for example for those
Supply of modified Konstrukte, which obtain new phänotypische properties, must those
DNA the landlord organisms insulated become. Only then it stands again for manipulation for purpose to < RTI ID=1.28> Verfügung.< /RTI> For further Vermehrung it must be brought again into suitable landlord organisms. Thus often many process steps are and/or < RTI ID=1.29> umständliche< /RTI> Manipulations necessarily, in order to
top produce a desired DNA. It is also unfortunately conceivable and the person skilled in the art well-known that this < itself; RTI ID=1.30> Aufwand< /RTI> still multiplies, if a larger number of different DNAs < RTI ID=1.31> hergestellt< /RTI> will is.

A further method for " in vitro " - synthesis of more doppelsträngiger, well-known in the state of the art
DNA is the PCR technology. A condition < RTI ID=1.32> für< /RTI> a such preparation is < RTI ID=1.33> Verfügbarkeit< /RTI> more suitably
Stencil DNA. < RTI ID=1.34> Subklonierung< /RTI> suitable DNA fragments and the perhaps lengthy The experimental works know adjustment of the correct reaction conditions for the PCR < RTI ID=2.1> beträchtlich< /RTI> retard.

Those managing described, in the state of the art admitted methods are still relatively zeitund thereby also < RTI ID=2.2> kostenaufwendig.< /RTI> Besides are they, as < in case of; RTI ID=2.3> CDNA Clonierung, < /RTI> not always easily successfully. The synthetic generation of < RTI ID=2.4> längeren< /RTI> Nucleinsäurefragmenten causes in practice often substantial difficulties. Also the generation of DNA by PCR, < RTI ID=2.5> obwohl< /RTI> it the DNA< RTI ID=2.6> Rekombinationstechnik< /RTI> far advanced, can in < RTI ID=2.7> Einzelfall< /RTI> not of < RTI ID=2.8> Erfolg< /RTI> < RTI ID=2.9> gekrönt< /RTI> its or on difficulties < RTI ID=2.10> push, < /RTI> as was managing described.

Task of the present invention was to make a method available which < the synthesis of Nucleinsäuremolekülen of desired sequence and Linge in simple and time-saving way; RTI ID=2.11> ermöglicht.< /RTI> This task is < by in; RTI ID=2.12> Ansprüchen< /RTI> characterized embodiments < RTI ID=2.13> gelöst.< /RTI>

The invention < RTI ID=2.14> betrifft< /RTI> thus a method to the synthesis of Nucleinsäuremolekülen, which < the following steps partly or; RTI ID=2.15> vollständig< /RTI> < RTI ID=2.16> umfasst< /RTI> : 1. Supply of a Nucleinsäuremoleküls, which exhibits at least an end, which < an accumulation and/or; RTI ID=2.17>

Verknüpfung< /RTI> of and/or. with a further Nucleinsäuremolekül < RTI ID=2.18> erlaubt< /RTI> ; 2. Accumulation and/or < RTI ID=2.19> Verknüpfung< /RTI> at least a further Nucleinsäuremoleküls to that and/or. with the Nucleinsäuremolekül, whereby end at least a further Nucleinsäure of the molecule to that and/or. with that at least (n) end of the Nucleinsäuremoleküls angelagert and/or < RTI ID=2.20> verknüpft< /RTI> becomes, and the other end at least a further Nucleinsäuremoleküls in
One falls < RTI ID=2.21> Verknüpfung< /RTI> masked is;
3. If necessary masking at least end of the Nucleinsäuremoleküls, to that and/or. with none further the Nucleinsäuremolekül angelagert and/or < RTI ID=2.22> verknüpft< /RTI> became;
4. Cracking at least a further angelagerten and/or linked < RTI ID=2.23> Nucleinsäuremoleküls< /RTI> to pre-determined steep ones, whereby masking is removed, and an end produced, that is < an accumulation and/or; RTI ID=2.24> Verknüpfung< /RTI> of and/or. with a further < RTI ID=2.25> Nucleinsäuremolekül permits; < /RTI> and
5. At least in, if necessary repeated repetition of the steps (2) to (4), whereby in step (2) suitable in each case Nucleinsäuremoleküle is used.

In a preferential embodiment < RTI ID=2.26> erfindungsgemässen< /RTI> Method is the further Nucleinsäuremolekül a Nucleinsäure Einzelstrangmolekül.

In a particularly preferred embodiment < RTI ID=2.27> umfasst< /RTI> < RTI ID=2.28> erfindungsgemässe< /RTI> Method after step (2) the following step: (2a) Replenishment of the second Nucleinsäurestrangs by a polymerase activity, complementary to the single strand in its sequence, whereby masking is removed if necessary before. In a further particularly preferential embodiment < RTI ID=3.1> umfasst< /RTI> < RTI ID=3.2> erfindungsgemässe< /RTI> Method after step (4) or (5) the following step: (4/5a) < RTI ID=3.3> Auffüllung< /RTI> the second Nucleinsäurestrangs by one, complementary to the single strand in his sequence, < RTI ID=3.4> Polymeraseaktivität. < /RTI>

As managing < RTI ID=3.5> mentioned, < /RTI> is suitable < RTI ID=3.6> erfindungsgemässe< /RTI> Method to the synthesis of more einzelsträngiger (dsDNA) or partiel < RTI ID=3.7> doppelsträngiger< /RTI> DNA.

The principle < RTI ID=3.8> erfindungsgemässen< /RTI> Method is in Fig. 1 < RTI ID=3.9> dargestellt.< /RTI> Further embodiments are in the Fig. 2 to 7 < RTI ID=3.10> dargestellt.< /RTI>

In an embodiment < RTI ID=3.11> erfindungsgemässen< /RTI> Method a einzelsträngiges Nucleinsäuremolekül is < RTI ID=3.12> partial doppelsträngiges Nucleinsäuremolekül< /RTI> with over-hanging 5 ' - or 3 ' - an end or < RTI ID=3.13> doppelsträngiges< /RTI> Nucleinsäuremolekül with a smooth end made available. To this end of the made available Nucleinsäuremoleküls in one < RTI ID=3.14> nächsten< /RTI> Step with < RTI ID=3.15> Hilfe< /RTI> one < RTI ID=3.16> Ligaseaktivität, < /RTI> for example bind for a T4-RNA-Ligase, a einzelsträngiges Nucleinsäuremolekül kovalent. < RTI ID=3.17> einzelsträngige Nucleinsäuremolekül< /RTI> can be bound thereby with his 5 ' - phosphate or 3 ' - hydraulic XY end to the made available Nucleinsäuremolekül. Accordingly < RTI ID=3.18> umfasst< /RTI> < RTI ID=3.19> erfindungsgemässe< /RTI> Method a Nucleinsäuresynthese in 3 ' - 5 ' - or in 5 ' - 3 ' - direction (of that Orientation of the forerunner molecules outgoing). In these embodiments it is substantially, < RTI ID=3.20> dass< /RTI> that
End of the einzelsträngigen Nucleinsäuremoleküls, which does not < with the made available; RTI ID=3.21> Nucleinsäure < /RTI> molecule is linked, masked is. Masked one means in this sense of the present invention that this end in this Ligationsansatz does not < with another einzelsträngigen Nucleinsäuremolekül of the same kind; RTI ID=3.22> verknüpft< /RTI> will can, and from it einzelsträngige molecules to result, those of several replicas of the same Nucleinsäuremoleküls to consist and likewise with the made available
Nucleinsäuremolekül < RTI ID=3.23> verknüpft< /RTI> are < RTI ID=3.24> können.< /RTI> In the sense of the invention a masking is a chemical, enzymatic or other modification of that end, that the o. g. < RTI ID=3.25> Verknüpfung< /RTI> prevented. Masking in the sense of this invention are described still more exactly in the following.

After the Ligation become the ends of the made available Nucleinsäuremoleküle masked, which < with none; RTI ID=3.26> einzelsträngigen< /RTI> Nucleinsäuremolekül < RTI ID=3.27> verknüpft< /RTI> are. < RTI ID=3.28> Im< /RTI> < RTI ID=3.29> nächsten< /RTI> Step is < the einzelsträngige Nucleinsäuremolekül, which was ligiert to the made available Nucleinsäuremolekül, in a pre-determined place split, whereby masking is removed and an end is produced, one; RTI ID=3.30> Verknüpfung< /RTI> with a next einzelsträngigen Nucleinsäuremolekül permits. By two the latter
Steps < RTI ID=3.31> gewährleistet< /RTI> < RTI ID=3.32> erfindungsgemässe< /RTI> Method in < RTI ID=3.33> vorteilhafter< /RTI> Way that in further
Ligationsschritten only those Nucleinsäuremoleküle of far elongated are <, to in; RTI ID=3.34> vorangegangenen< /RTI> Walked a einzelsträngiges Nucleinsäuremolekül was ligiert. The Ligations, Maskierungs-und < RTI ID=3.35> Spaltungsschritt< /RTI> can arbitrarily often be repeated in this sequence with again in each case molecules which can be deposited now, whereby suitable in each case einzelsträngige Nucleinsäuremoleküle is used. In another preferential embodiment < RTI ID=4.1> erfindungsgemässen< /RTI> Method one synthesizes after the synthesis of the complete desired single strand of the Gegenstrang with a polymerase activity, complementary in his sequence. One < RTI ID=4.2> Einzelstrang< /RTI> in 3 ' - 5 ' - direction synthesized and a doppelsträngiges Nucleinsäuremolekül with a smooth end or a partial doppelsträngiges Nucleinsäuremolekül was made available, can be synthesized the complementary Nucleinsäurestrang directly of the Nucleinsäuremoleküls made available by the free 3 ' - end. However a einzelsträngiges Nucleinsäuremolekül was < RTI ID=4.3> bereitgestellt< /RTI> and the synthesis takes place < RTI ID=4.4> Einzelstrangs< /RTI> in 3 ' - 5 ' - direction, < RTI ID=4.5> muss< /RTI> < RTI ID=4.6> über< /RTI> Hybridizing a suitable einzelsträngigen Nucleinsäureoligomers to the made available Nucleinsäuremolekül before the polymerase reaction a 3 ' end to < RTI ID=4.7> Verfügung< /RTI> are placed. If the synthesis of the Nucleinsäureeinzelstrangs took place in 5 ' - 3 ' direction, becomes the last einzelsträngige Nucleinsäuremolekül, which < at the synthesized; RTI ID=4.8> Nucleinsäure < /RTI> < RTI ID=4.9> einzelstrang< /RTI> one ligiert, favourable-proves in such a way < RTI ID=4.10> selected, < /RTI> < RTI ID=4.11> dass< /RTI> the 3 ' - end a hairpin structure trains, then < RTI ID=4.12> dass< /RTI> after cracking of the hairpin structure a 3 ' - end for the synthesis of the complementary < RTI ID=4.13> Nucl einsäurestranges< /RTI> by a polymerase activity to < RTI ID=4.14> Verfügung< /RTI> one places.

In a further preferential embodiment < RTI ID=4.15> erfindungsgemässen< /RTI> Method one < directly after (everyone) cracking of the ligierten einzelsträngigen Nucleinsäuremoleküls before the Ligation; RTI ID=4.16> nächsten< /RTI> einzelsträngigen Nucleinsäuremoleküls the appropriate complementary Nucleinsäurestrang by means of a polymerase activity synthesizes. < RTI ID=4.17> Im< /RTI> substantial one proceeds otherwise thereby as described managing for the synthesis of the complete complementary Nucleinsäurestrangs. < RTI ID=4.18> Im< /RTI> One falls < RTI ID=4.19> 5 ' - 3 ' < /RTI> - Synthesis direction is < in such a way thereby each Nucleinsäure Einzelstrangmolekül; RTI ID=4.20> gewählt, < /RTI> < RTI ID=4.21> dass< /RTI> it at the 3 ' end < RTI ID=4.22> vorteilhafterweise< /RTI> a hairpin structure trains. < RTI ID=4.23> Zusätzlich< /RTI> before one < RTI ID=4.24> Polymerase reaction, < /RTI> before masking the ends of the made available < RTI ID=4.25> Nucleinsäuremoleküle< /RTI> taken place, masking to < RTI ID=4.26> 3 ' - < /RTI>
End of the ligierten Nucleinsäuremoleküls removes.

In a further embodiment < RTI ID=4.27> erfindungsgemässen< /RTI> Method are the further Nucleinsäuremoleküle, which < to the made available Nucleinsäuremolekül angelagert and/or thus; RTI ID=4.28> verknüpft< /RTI> become doppelsträngig. In this embodiment the made available Nucleinsäuremolekül is einzelsträngig or partiel doppelsträngig with a over-hanging 3 ' - or 5 ' - end. If the further Nucleinsäuremolekül an appropriate, in its sequence complementary possesses, < RTI ID=4.29> überhängendes< /RTI>
3 ' - or 5 ' - end, finds an accumulation by hybridizing the einzelsträngigen over-hanging
Ends instead of. Preferably the other end of the further Nucleinsäuremoleküls is smooth. By those managing described masking is < RTI ID=4.30> ensured, dass< /RTI> to this end no accumulation < RTI ID=4.31> über< /RTI> Hybridizing cohesive ends of further Nucleinsäuremolekülen of the same kind takes place.

In again different embodiment < RTI ID=4.32> erfindungsgemässen< /RTI> Method is the further Nucleinsäuremolekül einzelsträngig and it finds an accumulation < to the made available Nucleinsäure molecule; RTI ID=4.33> über< /RTI> Hybridizing of complementary finalconstant nucleotides instead of. In this
Embodiment is einzelsträngig or partiel doppelsträngig < with a over-hanging the made available Nucleinsäuremolekül; RTI ID=4.34> 3 ' - oder< /RTI> 5 ' - end. If necessary the einzelsträngige further can Nucleinsäuremolekül < RTI ID=4.35> zusätzlich< /RTI> kovalent with the made available < RTI ID=4.36> Nucleinsäuremolekül< /RTI> by means of one < RTI ID=5.1> Ligaseaktivität to be linked. If hybridizing takes place over 3 ' - finalconstant nucleotides, kann< /RTI> in < RTI ID=5.2> nächsten< /RTI> Step by means of a polymerase activity the complementary strand to be synthesized. Hybridizing finds < RTI ID=5.3> über< /RTI> < RTI ID=5.4> 5-endständige< /RTI> Nucleotides instead of, that becomes 3 ' - end of the further einzelsträngigen Nucleinsäuremoleküls < in such a way; RTI ID=5.5> selected, < /RTI> that it one < RTI ID=5.6> Haarnadelstruktur< /RTI> trains, so that a 3 ' - end for < RTI ID=5.7> anschliessende< /RTI> Polymerization reaction is made available to the synthesis of the complementary Nucleinsäurestrangs. In the next step the synthesized Nucleinsäuredoppelstrang in a pre-determined place is split, whereby the recognition sequence necessary for the cracking and the smooth end and/or. < RTI ID=5.8> Haarnadelstruktur< /RTI> is removed and a preferably cohesive end develops, which < one; RTI ID=5.9> Accumulation über< /RTI> Hybridizing and a kovalente < if necessary; RTI ID=5.10> Verknüpfung< /RTI> the Nucleinsäuremoleküls with a further einzelsträngigen Nucleinsäuremoleküls < RTI ID=5.11> erlaubt.< /RTI>

The present invention < RTI ID=5.12> umfasst< /RTI> in addition methods, whose accumulation, Maskierungs-und </or splitting steps combinations of the appropriate steps of the aforementioned embodiments; RTI ID=5.13> darstellen.< /RTI> So can for example in a first synthesis cycle < RTI ID=5.14> einzelsträngiges Nucleinsäure < /RTI> molecule kovalent with a made available Nucleinsäuremolekül < RTI ID=5.15> verknüpft< /RTI> become, < RTI ID=5.16> anschliessend< /RTI> the complementary Nucleinsäurestrang to be synthesized, doubling rank as be managing described split, and in < RTI ID=5.17> nächsten< /RTI> Synthesis cycle a further einzelsträngiges Nucleinsäuremolekül < RTI ID=5.18> über< /RTI> Hybridizing to be angelagert.

Einzelsträngige Nucleinsäuremoleküle is < by means of one; RTI ID=5.19> Ligaseaktivität< /RTI> with a made available < RTI ID=5.20> Nucleinsäuremolekül linked, muss< /RTI> the synthesis of the complementary Nucleinsäurestrangs after everyone does not < RTI ID=5.21> Accumulation, < /RTI> Maskierung-und/or < RTI ID=5.22> Spaltungsschritt< /RTI> or at the end of the synthesis of the complete Nucleinsäureeinzelstrangs take place. The time of filling up the complementary strand can arbitrarily < RTI ID=5.23> gewählt< /RTI> it becomes, in the sense that he < for example after a any permutating accumulation, Maskierungs-und/or splitting step; RTI ID=5.24> gewählt< /RTI> becomes.

< RTI ID=5.25> Term, masking " bedeutet< /RTI> in the sense of the present invention, < RTI ID=5.26> dass< /RTI> a kovalente < RTI ID=5.27> Verknüpfung< /RTI> two Nucleinsäuremoleküle does not < RTI ID=5.28> möglich< /RTI> is. Masked einzelsträngige 3 ' - ends can do for example by the installation of a Aminoblocks, a Didesoxynucleotids, one < RTI ID=5.29> 3 ' - Phosphats< /RTI> or by a synthetic inserted 5 ' - end to be produced. Masked einzelsträngige 5 ' - ends draw in the sense of the present invention for example by a missing group of phosphates or by the installation of 5 ' - modified nucleotide (z. B. Biotin dNTP, Digoxygenin dNTP) out. One finds
Extension of a made available Nucleinsäuremoleküls < RTI ID=5.30> über< /RTI> Hybridizing complementary, finalconstant nucleotides instead of, then becomes a doppelsträngiges Nucleinsäuremolekül with a smooth end, to which no further Nucleinsäuremolekül with its finalconstant nucleotides hybridize < RTI ID=5.31> can, im< /RTI>
Senses of the present invention also as masked designates. Partiel a doppelsträngiges Nucleinsäuremolekül also over-hanging einzelsträngigen ends can become thus masked, by < RTI ID=5.32> überhängendes 3 ' - end mittels< /RTI> for a Exonucleaseaktivität one removes, or which complementary strand is synthesized to over-hanging 5 ' - an end by means of a polymerase activity, so that in both < RTI ID=5.33> Fällen< /RTI> a doppelsträngiges Nucleinsäuremolekül with smooth ends develops. < RTI ID=6.1> Term., < /RTI> < RTI ID=6.2> Bereitstellen< /RTI> one < RTI ID=6.3> Nucleinsäuremolekülś " umfasst< /RTI> any form < RTI ID=6.4> Making available, < /RTI> z. B. the Clonierung gene with < RTI ID=6.5> anschliessender< /RTI> Restriction splitting and < RTI ID=6.6> Isolierung< /RTI> a fragment with z. B. a over-hanging or smooth end, which < as starting material for; RTI ID=6.7> erfindungsgemässe< /RTI> Method serves. In another implementing us form the Nucleinsäuremolekül is made available by Aneinanderlagerung by two at least partial complementary synthetic Oligonucleotiden, whereby by < RTI ID=6.8> Aneinanderlagerung< /RTI> < RTI ID=6.9> Überhang< /RTI> to develop can. In a further embodiment

einzelsträngige Oligonucleotide is made available.

< RTI ID=6.10> Term " an< /RTI> at least an end ", as < RTI ID=6.11> erfindungsgemäss< /RTI> used, it means that the synthesis can run university or bi-directional.

< RTI ID=6.12> The " accumulation " the Nucleinsäure Einzelstrangmoleküle erfolgt< /RTI> preferably by hybridizing. The necessary hybridizing conditions can become, if necessary, by the person skilled in the art easily for each step of the accumulation of a new single strand from its expertise modified.

In < RTI ID=6.13> erfindungsgemässen< /RTI> Methods assigned Nucleinsäure Einzelstrangmoleküle have one Length of maximally approx. 150 < RTI ID=6.14> Nucleotiden.< /RTI> Preferentially a length between 15 and 130 is < RTI ID=6.15> Nucleotiden.< /RTI> Generally is < with; RTI ID=6.16> Wahl< /RTI> the length of the single strand molecules to consider, < RTI ID=6.17> dass< /RTI> the yield intact
Oligonucleotide with the chemical synthesis of single strand precursor molecules with more increasingly Length sinks because of incorrect installation of nucleotides. It is thus < RTI ID=6.18> Kompromiss< /RTI> to be received between Linge of the Oligonucleotide and their yield. An influence on the yield to desired Nucleinsäure with the method according to invention also < RTI ID=6.19> Qualität< /RTI> for the those
Synthesis of assigned single strand molecules. By the Oligonucleotidreinigung with < RTI ID=6.20> Hilfe< /RTI> the HPLC are intact the individual Nucleinsäure Einzelstrangmoleküle for resuming syntheses. Finally the length of the Oligonucleotide used in resuming syntheses becomes after that
Quantity need for a synthesis step and the yield with the chemical synthesis orient.

< RTI ID=6.21> Term " pre-determined place ", < /RTI> how it uses according to invention means that this sequence < either by its primary sequence or by their relative positioning to the actual; RTI ID=6.22> Spaltungsstelle< /RTI> is defined.

A pre-determined place to the cracking of a Nucleinsäureeinzelstrangs can for example through < RTI ID=6.23> Inkorporation< /RTI> one or several more artifizieller or more modified < RTI ID=6.24> Nucleotides, < /RTI> Cousin analogues or a chemical group, internally or terminals, to be produced,/by means of a physical, chemical or enzymatic method be split knows, so that < RTI ID=6.25> 3 ' - OH-and/oder< /RTI> < RTI ID=6.26> 5 ' - < /RTI> Phosphate and develops (z. B. Maxam Gilbert reaction etc.). Nucleotides, which for < itself; RTI ID=6.27> erfindungsgemässe< /RTI> Methods are suitable, are for example 5-Hydroxy-2-desoxycytidin, 5-Hydroxy-2 desoxyuridin or < RTI ID=6.28> 5-Hydroxy-2' desoxyuridin.< /RTI> While the first two nucleotides of substrates for E. Coli Endonuclease III and Formamidopyrimidine DNA < RTI ID=6.29> Glycosylase< /RTI> < RTI ID=6.30> represent, < /RTI> can at latter nucleotide by means of < RTI ID=6.31> Uracil DNA Glycosylase< /RTI> and Apyrimidase and/or. Alkali treatment to be split. For example can < RTI ID=7.1> Phosphoborannukleotide< /RTI> or Thioatnukleotide in the DNA sequence a terminal digest from < RTI ID=7.2> Exonuklease II< /RTI> or T7 (gene 6) < RTI ID=7.3> Nuklease< /RTI> bring to the stop, whereby the sequence up to the modified < RTI ID=7.4> Nukleotid< /RTI> to the cracking is pre-determined.

A further < RTI ID=7.5> Möglichkeit< /RTI> to the cracking of the Nucleinsäureeinzelstranges in a pre-determined place exists in < RTI ID=7.6> Einführung< /RTI> < RTI ID=7.7> a " mismatches " in< /RTI> a artifiziellen hairpin structure. Composition one < RTI ID=7.8> ?mismatch< /RTI> repair " - enzyme < RTI ID=7.9> lässt< /RTI> this structure efficiently and < itself; RTI ID=7.10> präzise< /RTI> split.

Which (molecular) agent as restriction activity to the cracking of one or several pre-determined < RTI ID=7.11> Stellen< /RTI> in a Nucleinsäuredoppelstrang in < RTI ID=7.12> erfindungsgemässen< /RTI> Method in the long run use finds, is not invention substantial. Substantially however it is for embodiments which cover the cracking of doppelsträngiger Nucleinsäure that, as already managing exemplary < RTI ID=7.13> mentioned, < /RTI> the recognition sequence on the Nucleinsäure and < RTI ID=7.14> tatsächlich< /RTI> split sequence from each other < RTI ID=7.15> örtlich< /RTI> are separate. < RTI ID=7.16> Erfindungsgemäss< /RTI> the recognition sequence is usually removed by the cracking from the increasing Nucleinsäure Doppelstrangmolekül. < RTI ID=7.17> Restriktionsendonucleasen< /RTI> the class properties, which correspond to the requirements to such an agent, possess II S. Are < depending upon embodiment; RTI ID=7.18> erfindungsgemässen< /RTI> Method representative of this class, which produce a free, cohesive 3 ' - end or a supernatant, cohesive 5 ' - end, suitably.

The properties < RTI ID=7.19> Restriction activities, < /RTI> in < RTI ID=7.20> erfindungsgemässen< /RTI> Methods applicable are < as follows, can; RTI ID=7.21> zusammengefasst< /RTI> become: (A) the restringierende agent can be various nature: in addition < RTI ID=7.22> gehören< /RTI> all Nucleinsäuren specifically < RTI ID=7.23> spaltenden< /RTI> synthetic agents like synthetic peptides, PNA (peptide nucleic acid), tripelhelikale DNA binding Oligonucleotide, those for the specific processing/that
Nucleinsäure Terminus/i in the sense of this invention are suitable, like also natural occurring DNA splitting enzymes. The person skilled in the art is in the situation, for its respective tack suitable (Exo) Nuklease and/or. also < RTI ID=7.24> Restriktionsaktivitäten< /RTI> to begin; (B) these can for example < RTI ID=7.25> Restriktionsendonucleasen< /RTI> the type II S its; (C) asymmetric recognition sequences < RTI ID=7.26> (Restriktionsendonucleasen< /RTI> the class II S), like also symmetric recognition sequences are applicable thereby; (D) like already managing < RTI ID=7.27> mentioned, < /RTI> may the gap places, which < by; RTI ID=7.28> Restriktionsaktivität< /RTI> produced, not within the specific recognition sequence to lie, but are < RTI ID=7.29> müssen< /RTI> < RTI ID=7.30> 5 ' or 3 ' distal< /RTI> located of it its; (E) the distance of the interface of the recognition sequence must be exact and clearly defined; < RTI ID=7.31> (f) < /RTI> < RTI ID=7.32> around the Spezifität< /RTI> the accumulation < RTI ID=7.33> Nucleinsäure Einzelstranges< /RTI> in an embodiment < RTI ID=7.34> erfindungsgemässen< /RTI> Method too < RTI ID=7.35> gewährleisten< /RTI> and an efficient Ligationsreaktion too < RTI ID=7.36> ensure, < /RTI> if this is desired, the Nukleaseaktivität produces and/or. the restringierende
Agent preferably < RTI ID=7.37> kohäsive< /RTI> Ends. Thus also those is void managing discussed necessity for masking the single strands, the z. B. to the smooth ends to be angelagert. A suitable selection agents restringierenden on can the person skilled in the art < RTI ID=8.1> beigefügten< /RTI> Bibliography take.

The lead-through of the step (5) and/or. < RTI ID=8.2> Häufigkeit< /RTI> its lead-through < RTI ID=8.3> hängt< /RTI> finally of the long one of the desired final product, and of the strand length to < RTI ID=8.4> Verfügung< /RTI> standing starting material off.

In another particularly preferred embodiment i. becomes. D. R. synthetic, einzelsträngiges DNA molecule (+) to < RTI ID=8.5> Verfügung< /RTI> placed, its 5 ' - Teminus for hybridizing with that 3 ' - end of the preceding templateabhängig synthesized < RTI ID=8.6> Einzelstrang DNA Moleküls< /RTI> (-) gebracht the free synthesizing barness of the DNA sequences is < many of the today usual methods of time-consuming DNA manipulations from the laboratory to the synthesis machine; RTI ID=9.1> shift, < /RTI> whereby a large costing, and with it connected large saving of time results. The experiment which can be accomplished by the person skilled in the art consists then of the Design of a Nucleinsäuresequenz at a computer editor and < RTI ID=9.2> Examination, < /RTI> whether by < itself; RTI ID=9.3> Sequenzmanipulationen< /RTI> desired properties at the biological model or in vitro adjust. < RTI ID=9.4> erfindungsgemässe< /RTI> Method is thus also a contribution for the advancement of techniques of the reversen genetics.

In a preferential embodiment < RTI ID=9.5> erfindungsgemässen< /RTI> Method into the made available Nucleinsäuremolekül inkorporierte further are < RTI ID=9.6> Nucleinsäuremoleküle, < /RTI> Fragments separated from it and/or nucleotides after step (2), (2a), (3), (4), (4a), (5) and/or (ä).

The separation of the inkorporierten < RTI ID=9.7> Nucleinsäure Einzeistrangmoleküle< /RTI> is preferential, but necessarily necessarily and cannot of the person skilled in the art after < RTI ID=9.8> Standard technique, < /RTI> z. B. through < RTI ID=9.9> säulenchromatographische Verfahren< /RTI> are managed. The concentration at free Nucleotidphosphaten < RTI ID=9.10> könnte< /RTI> in particular for the Gesamtausbeute to desired Nucleinsäure limiting, spent nucleotides the synthesis and Ligationsreaktion are < RTI ID=9.11> disturb, < /RTI> the z. B. in case of the generation of smooth ends and/or. the accumulation of Nucleinsäure Einzelsträngen to smooth
Ends and following production of the complementary strand with the lead-through < RTI ID=9.12> erfindungsgemässen< /RTI> Method is necessary, as was managing described. A high
Concentration of different Einzelstrang DNAs < RTI ID=9.13> erhöht< /RTI> the risk of unwanted by-products.

Practical it is of advantage therefore, if each individual synthesis step under optimal conditions < RTI ID=9.14> ablaufen< /RTI> can. Thus < RTI ID=9.15> empfiehlt< /RTI> a separation that does not < itself; RTI ID=9.16> benötigten< /RTI> Single strands before < in each case; RTI ID=9.17> nächsten< /RTI> Synthesis step, for example in a matrix-coupled reaction, at whose expiration of spent nucleotides and surplus Einzelstrang Nucleinsäuren < RTI ID=9.18> eluiert< /RTI> become. The separation can take place thus naturally also after or during the lead-through of the step (5).

Those managing described optional Ligation in the embodiment < RTI ID=9.19> erfindungsgemässen< /RTI> Method, which < the accumulation; RTI ID=9.20> über< /RTI> Hybridizing complementary finalconstant nucleotides enclosure, can take place for example forwards, simultaneous with or after step (4). In another embodiment it can take place after or during the step (5). An example of the Ligation after step (5) supplies the case that bacteria, z. B. E. coli, with the ligierten synthesis product to be transformed and those
Ligation by endogenous ligases is made. Like that it is well-known that with increasing value < RTI ID=9.21> complementary lap kohäsiver< /RTI> Ends a transformation for example of E. coli with suitable DNA under utilization the endogenous < RTI ID=9.22> Ligaseaktivität< /RTI> to the Zirkularisierung < RTI ID=9.23> möglich< /RTI> is. Are < RTI ID=9.24> Lücken< /RTI> and supernatant Einzelstrang DNAs quite tolerates, since repair mechanisms < RTI ID=9.25> Integrität< /RTI> circular letters of doubling rank repair. Single strand ranges are < RTI ID=9.26> aufgefüllt< /RTI> and repairs, if at least a Phosphodiesterrückgrat is intact. Preferably a Ligation is made if the overhangs are long only few nucleotides. < RTI ID=9.27> In case of longer Überhänge< /RTI> it is conceivable that between the finalconstant nucleotides Einzel-und of doubling rank < RTI ID=9.28> Lücken< /RTI> arise, which become closed before a Ligationsreaktion for example by a polymerase activity. Since those so far admitted restriction enzymes mostly only relatively short < RTI ID=10.1> kohäsive< /RTI> Ends produce, are also a C and a G and/or. A and < RTI ID=10.2> T " tailing " with terminaler< /RTI> Transferase conceivably, which < for a long time; RTI ID=10.3> Lap ranges produces, which are transformed directly without " into vitro " Ligation können.< /RTI> Finally the synthesis product can also after step of the isolation of the finished synthesis product of a Ligation < RTI ID=10.4> zugeführt< /RTI> become.

As already managing < RTI ID=10.5> mentioned, < /RTI> in a preferential embodiment one < RTI ID=10.6> erfindungsgemässen< /RTI> Method the pre-determined place of the Nucleinsäuremoleküles through < RTI ID=10.7> Inkorporation< /RTI> one < RTI ID=10.8> artifiziellen< /RTI> or modified nucleotide, one < RTI ID=10.9> Cousin analogues, < /RTI> a chemical group or < RTI ID=10.10> " bad match " < /RTI> in a artifiziellen hairpin structure one produces,//by means of a physical, chemical or enzymatic method to be split can.

In a particularly preferred embodiment the artifizielle or < is; RTI ID=10.11> modifizierte< /RTI> Nucleotide 5 Hydroxy-2-desoxy-cytidin, 5-Hydroxy-2-desoxyuridin, or < RTI ID=10.12> 5-Hydroxy-2' desoxyuridin.< /RTI>

As already likewise managing < RTI ID=10.13> mentioned, < /RTI> < RTI ID=10.14> betrifft< /RTI> the present invention in a further preferential embodiment a method, with which < RTI ID=10.15> Verknüpfung< /RTI> of two finalconstant nucleotides over
3 ' - Hydroxy-und < RTI ID=10.16> 5 ' - phosphate Ende< /RTI> with < RTI ID=10.17> Hilfe< /RTI> one < RTI ID=10.18> Ligaseaktivität, < /RTI> and the accumulation < RTI ID=10.19> über< /RTI> those
Hybridizing complementary sequences take place.

In another preferential embodiment < RTI ID=10.20> erfindungsgemässen< /RTI> Method is the Nucleinsäure DNA. In a further preferential embodiment < RTI ID=10.21> erfindungsgemässen< /RTI> Method is those Nucleinsäure RNA. Of < RTI ID=10.22> erfindungsgemässen< /RTI> Method < RTI ID=10.23> umfasst< /RTI> is also the generation of
DNA/RNA hybrid.

In a further preferential embodiment < RTI ID=10.24> erfindungsgemässen< /RTI> Method takes place those Masking in step (3) additive and substraktiv by adding and/or. Distance of a chemical Group or a chemical < RTI ID=10.25> Moleküls.< /RTI> In a preferential embodiment < RTI ID=10.26> erfindungsgemässen< /RTI> Method takes place masking 5 ' - an end via removing the group of phosphates (n) or that Installation 5 ' - of a modified nucleotide (z. B. Biotin dNTP, Digoxygenin dNTP etc.). As managing < RTI ID=10.27> mentioned, < /RTI> becomes by a masking 5 ' - end one which can be deposited < RTI ID=10.28> Nucleinsäure < /RTI> Single strand molecule in the appropriate embodiment < RTI ID=10.29> erfindungsgemässen< /RTI> Method an unwanted Ligasenebenreaktion between the 5 ' - and 3 ' - ends < RTI ID=10.30> Nucleinsäure < /RTI> Single strand molecules among themselves prevented and thus < RTI ID=10.31> möglichen< /RTI> Formation of Konkatemeren prevented, whereby an optimal yield of the method < RTI ID=10.32> erfindungsgemässen< /RTI> Teachings < RTI ID=10.33> gewährleistet< /RTI> becomes.

In a particularly preferred embodiment of the method according to invention those takes place Masking by the installation at least 5 ' - a modified nucleotide. In a further preferential embodiment < RTI ID=11.1> erfindungsgemässen< /RTI> Method draws, as already < RTI ID=11.2> mentioned, < /RTI> masked 3 ' - end by the presence of a Aminoblocks, a Didesoxynucleotids, a 3 ' - phosphate, or one < RTI ID=11.3> künstlichen< /RTI> < RTI ID=11.4> 5 ' - Endes< /RTI> out.

In a further preferential embodiment the further Nucleinsäuremolekül at the bereitgestellten Nucleinsäuremolekül forms after accumulation and/or < RTI ID=11.5> Verknüpfung< /RTI> removed end one < RTI ID=11.6> Haarnadelschleife< /RTI> out, which serves as primer for the polymerase activity.

The invention relates to in a further preferential embodiment a method, whereby the cracking in a pre-determined place in step (4) sequence-specifically < by one; RTI ID=11.7> spaltende< /RTI> tripelhelikale DNA takes place.

A tripelhelikale DNA becomes z. B. in an educated manner if one < itself; RTI ID=11.8> Einzelstrang DNA, < /RTI> at their end < RTI ID=11.9> Schwermetall< /RTI> (SM), to a DNA Doppelstrang deposits themselves and, if the sequence conditions are suitable, a tripelhelikale structure with one is coupled < RTI ID=11.10> DNA Doppelstrang< /RTI> trains.

The Nucleinsäure Doppelstrang is < of; RTI ID=11.11> Schwermetall< /RTI> at a defined position split.

< RTI ID=11.12> Darüber< /RTI> outside can as already < RTI ID=11.13> mentioned, < /RTI> < RTI ID=11.14> erfindungsgemäss< /RTI> each specific physical, chemical and enzymatic Nucleinsäurespaltung to be used, which < for the accumulation of a Nucleinsäure Einzelstrangmoleküles to the following Ligation with; RTI ID=11.15> Nucleinsäure < /RTI> Doubling rank molecule is favorable. Further examples < RTI ID=11.16> hierfür< /RTI> methodical are < RTI ID=11.17> Ansätze< /RTI> based on designten peptides or PNA (peptide nucleic acid). One < RTI ID=11.18> Übersicht< /RTI> < RTI ID=11.19> über< /RTI> for the person skilled in the art of the following bibliography can take the aforementioned molecules and examples from their application type.

Another preferential embodiment of the invention < RTI ID=11.20> betrifft< /RTI> a method, with which the cracking in a pre-determined place in step (4) < by type a II S; RTI ID=11.21> Restriktionsendonuclease< /RTI> taken place. Type or Class II S of enzymes possess an asymmetric, thus nichtpalindromische recognition sequence. < RTI ID=11.22> Gap places liegen< /RTI> either 5 ' - or 3-distal to the recognition sequence. Are < either; RTI ID=11.23> 5 ' - < /RTI> (z. B. < RTI ID=11.24> BspMI) < /RTI> or < RTI ID=11.25> 3 ' - < /RTI> (z. B. RleAI) supernatant ends or smooth ends (z. B. < RTI ID=11.26> BsmFI) < /RTI> produced.

In a particularly preferred embodiment < RTI ID=11.27> erfindungsgemässen< /RTI> Method is < those type; RTI ID=11.28> II< /RTI> S < RTI ID=11.29> Restriktionsendonuclease< /RTI> < RTI ID=11.30> RleAI Enzym< /RTI> from Rhizobium < RTI ID=11.31> leguminosarum< /RTI> (Vesely Z., < RTI ID=11.32> Müller< /RTI> A, Schmitz G, Kaluza K, Jarsch M, Kessler C (1990) R1eAI: A novel < RTI ID=11.33> clashs lys< /RTI> restriction endonuclease from Rhizobium < RTI ID=11.34> leguminosarum< /RTI> recognizing 5 ' - CCCACA (N12/9) - 3 ', genes 95: 129-131).

In another preferential embodiment < RTI ID=11.35> erfindungsgemässen< /RTI> Method is/is that Nucleinsäure Doppelstrangmoleküle and/or the Nucleinsäure Einzelstrangmoleküle of synthetic or semisynthetischen origin, whereby for the synthesis the inset of synthetic single strand molecules is particularly preferential. Semisyntheti molecules are producible by the fact that Nucleinsäurefragmente < RTI ID=12.1> aux, in< /RTI> vivo " (bacteria, yeast) amplifizierter DNA (dsDNA, ssDNA) or RNA in one or more intermediate steps < RTI ID=12.2> erfindungsgemässen< /RTI> Synthesis in defined places by Ligationsreaktionen to be inserted. This strategy knows in individual cases costs < RTI ID=12.3> beträchtlich< /RTI> to reduce help. For example as starter molecule the Nucleinsäuremolekül can likewise < RTI ID=12.4> " in< /RTI> vivo " produced DNA molecule its, to which by recursive DNA synthesis < RTI ID=12.5> beliebige< /RTI> DNA sequences < RTI ID=12.6> angehängt< /RTI> become.

In a further preferential embodiment of the method according to invention the synthesis is at least partly automated. So knows for example in a Nucleinsäure (towards) synthesis automats for < RTI ID=12.7> Nucleinsäure Doppelstränge< /RTI> from Nucleinsäure Einzelsträngen a battery of automated chemical < RTI ID=12.8> Oligonucleotidsynthesen< /RTI> (one already < in; RTI ID=12.9> grossem< /RTI> < RTI ID=12.10> Ausmasse< /RTI> practiced technology) the raw material for the synthesis of biological active, doppelsträngigen DNA molecules (z. B.) supply with to whole genes. These are < from the chemically synthesized Oligonucleotiden in a likewise automated method; RTI ID=12.11> hergestellt.< /RTI>

The Doppelstrangnucleinäuren which can be extended is bound at the synthesis matrix in a synthesis chamber. In this synthesis chamber run in one < RTI ID=12.12> zyklischen< /RTI> Reaction sequence again and again the same steps described above off. < RTI ID=12.13> Reaktionsnebenprodukte< /RTI> the preceding

Reaction are washed before beginning of a new reaction from the synthesis chamber. Around < RTI ID=12.14> Nucleinsäuremolekül elongated Startermolekül< /RTI> remains bound to the synthesis matrix. With everyone Synthesis step is inserted a Nucleinsäure with another sequence sequence, so that finally a if necessary doppelsträngige Nucleinsäure with the desired Nucleotidsequenz develops.

The invention relates to in a particularly preferred embodiment a method, with that those Synthesis matrix-bound < RTI ID=12.15> durchgeführt< /RTI> becomes.

All carrier materials, to which a Nucleinsäure can be bound and whose properties with the recursive Nucleinsäure synthesis desired are compatible, are applicable as synthesis matrix, z. B. < RTI ID=12.16> streptavidinbemantelte surfaces, < /RTI> whereby as starter molecule the used < RTI ID=12.17> Nucleinsäure < /RTI> Doubling rank molecule over an inserted biotinyliertes nucleotide is coupled to the synthesis matrix.

Further preferential Synthesematrices < RTI ID=12.18> schliessen< /RTI> Nylon surfaces, to which < RTI ID=12.19> polydT haltige< /RTI> Sequences by UV irradiation to be coupled, as well as tosyl, aktivester or epoxy-activated < RTI ID=12.20> Oberflächen< /RTI> (z. B.

GOPS), whereby the connection preferably < RTI ID=12.21> über< /RTI> < RTI ID=12.22> one, sAminolink " effected, < /RTI> like glass (CPG, fibre glass etc.), silicate, latex, polystyrene, epoxy or silicon.

In another preferential embodiment < RTI ID=12.23> erfindungsgemässen< /RTI> Method becomes the synthesized Nucleinsäuremolekül insulated after the synthesis.

This is done on the one hand via installation of an affinity-obtaining agent in the last synthesis step, like z. B. Biotin, Digoxiginin, Histidin tags or one < RTI ID=12.24> Maltoserestes.< /RTI> The so labeled Synthesis final products can thus simply and economically by means of more appropriate < RTI ID=13.1> Säulen< /RTI> insulated become.

Alternatively in the last synthesis step one < RTI ID=13.2> erfindungsgemässen< /RTI> Method < RTI ID=13.3> Plasmid< /RTI> with the synthesis product < RTI ID=13.4> verknüpft< /RTI> and the Nucleinsäuremolekül, if necessary after its Rezirkularisierung in bacteria, resulting from it, < RTI ID=13.5> eingeführt< /RTI> and < RTI ID=13.6> vervielfältigt.< /RTI> Alternatively into the made available Nucleinsäuremolekül as well as into the Nucleinsäuremolekül used in the last synthesis step defined sequences are inkorporiert, to the primer specifically to bind to be able. With < RTI ID=13.7> Hilfe< /RTI> these primers can the finished synthesized Nucleinsäuremolekül in a PCR reaction be amplifiziert, whereby the synthesis product of < RTI ID=13.8> Affinitätsmatrix< /RTI> insulated becomes. Are in the terminal sequences motives for sequence (z. B. < RTI ID=13.9> TypiIS< /RTI> Recognition places), by means of those < RTI ID=13.10> kohäsive< /RTI> Ends to be produced can, then it is easily possible to synthesize from individual DNA molecules such from still higher order to.

In another preferential embodiment < RTI ID=13.11> erfindungsgemässen< /RTI> Method become Nucleinsäure Einzelstrangmoleküle by denaturing the Nucleinsäure Doppelstrangmoleküls insulated.

This embodiment < RTI ID=13.12> erfindungsgemässen< /RTI> Method is in addition suitably, < RTI ID=13.13> Nucleinsäure < /RTI> < RTI ID=13.14> Einzelstrangmoleküle< /RTI> to manufacture arbitrary composition. In this connection particularly to mention is < RTI ID=13.15> Possibility, < /RTI> to make available such RNA molecules.

< RTI ID=13.16> Schliesslich< /RTI> the invention concerns a kit, comprising: (A) a ligase, and/or (B) a polymerase, (C) if necessary. < RTI ID=13.17> Type II< /RTI> S-Restriktionsenzym, (D) if necessary. one < RTI ID=13.18> Uracil DNA Glycosylase< /RTI> and a Apyrimidase and/or a one < RTI ID=13.19> Endonuclease< /RTI> III and one Formamidopyrimidin DNA < RTI ID=13.20> Glycosylase< /RTI> and/or a " bad match repair " enzyme, (E) a Phosphatase, a terminal transferase and/or one < if necessary; RTI ID=13.21> Exonuklease, < /RTI> if necessary a wash buffer to the Eluation of reaction by-products and not into that
Product < RTI ID=13.22> erfindungsgemässen< /RTI> If necessary synthesis inserted material, (g) a synthesis matrix with if necessary already to it a bound < RTI ID=13.23> Nucleinsäuremolekül as starter molecule, < /RTI> (h) if necessary suitable reaction buffers < RTI ID=13.24> für< /RTI> in (A) until (E) < RTI ID=13.25> aufgeführten< /RTI> Enzymes.

Due to the theory of the present invention as well as due to < RTI ID=13.26> allgemeinen< /RTI> Specialized knowledge in this technical field is < RTI ID=13.27> Hersteller< /RTI> < RTI ID=13.28> erfindungsgemässen< /RTI> Kits admits, how it < RTI ID=13.29> einzelnen< /RTI>
Components of the kit, z. B. the buffers, < RTI ID=13.30> herstellt< /RTI> and formulates. If necessary can < RTI ID=13.31> erfindungsgemässe< /RTI> Kit also a not to a matrix bound < RTI ID=13.32> Startermolekül< /RTI> and/or a sentence of suitable single strand molecules contain.

Description of the figures < RTI ID=14.1> Figure 1. ?In vitro? - ssDNA synthesis in 3 ' - 5 ' - direction (1) an< /RTI> a matrix coupled < RTI ID=14.2> Startermolekül< /RTI> (n) by means of one one < RTI ID=14.3> Ligaseaktivität um< /RTI> a n+1tes < RTI ID=14.4> Einzelstrangmolekül< /RTI> by one < RTI ID=14.5> 3 ' - < /RTI> < RTI ID=14.6> Die< links 5 Phosphodiesterbindung; /RTI> n+1te ssDNA possesses terminal < RTI ID=14.7> Uracildesoxynukleotid< /RTI> The glycosidische connection of the cousin < RTI ID=14.8> Uracil< /RTI> by the DNA Uracilglycosylase it is split whereby a apyrimidinische position develop-developing this again < by a apyrimidinische; RTI ID=14.9> Endonukleaseaktivität< /RTI> < RTI ID=14.10> (ExonukleaseIII) < /RTI> so split that 5 ' - phosphate and < RTI ID=14.11> 3 ' - OH< /RTI> end develops. (3) to the freed < RTI ID=14.12> 5 ' - Phosphatende< /RTI> in the n+2ten Ligationsreaktion the n+2te is < RTI ID=14.13> SsDNA Molekul< /RTI> links a following phosphate reaction (not shown, s Figur4.) inactivates all DNA chains < RTI ID=14.14> für< /RTI> the n+3ten Ligationsschritt for all following Ligationsschritte, if no n+2tes < RTI ID=14.15> SsDNA Molekul< /RTI> in the n+2ten step was built.

By the DNA Uracilglycosylase and the apyrimidinische < RTI ID=14.16> Endonukleaseaktivität< /RTI> knows by processing 5 ' Phospahat for the next reaction sequence (n+3) to < RTI ID=14.17> Verfügung< /RTI> placed all steps repeat themselves k-times to the latter < RTI ID=14.18> SsDNA Molekul< /RTI> in m-ten step one inserted.

Figure 2. ?Into vitre? - dsDNA synthesis in 3 ' - 5 ' - direction all steps take place as in figure 1. , then however in the

last step on the basis of one < RTI ID=14.19> 3 ' - Ende< /RTI> < RTI ID=14.20> Startermoleküls< /RTI> started a polymerization reaction, the again synthesized single strand to doubling rank fills up alternative can in first and in the last step in each case a primer of a pair of primers be built and thus a doubling rank molecule through < RTI ID=14.21> Amplifikation< /RTI> by means of the PCR reaction to be produced.

?Invitro? - dsDNA-Synthesein3'-5' Richtung.AlleFigur3.

Steps take place as in figure one within each synthesis cycle after are < RTI ID=14.22> Phosphatasebehandlung< /RTI> < RTI ID=14.23> Polymerisationschritt< /RTI> introduced, that < RTI ID=14.24> anligierte< /RTI> ssDNA into one dsDNA < RTI ID=14.25> converts. The Prozessierung< /RTI> can also by means of one < RTI ID=14.26> Restriktionsendunuklease< /RTI> the type read take place, if a recognition sequence < built in each of the ssDNA fragments; RTI ID=14.27> vorliegt.< /RTI>

Figure < RTI ID=14.28> 4. ?In vitro? - dsDNA Synthese< /RTI> in < RTI ID=14.29> 3 ' - 5 ' - Richtung< /RTI> All steps take place as < in figure; RTI ID=14.30> 1 Eine< /RTI> after a Ligationsschritt durchgeF2hrte phosphate reaction inactivates all < RTI ID=14.31> DNA molecules für< /RTI> < RTI ID=14.32> nächsten< /RTI> Ligationsschritt and for all following Ligationsschritte, if none < RTI ID=14.33> SsDNA Molekul< /RTI> in synthesis cycle were inserted by the DNA Uracilglycosylase and the apyrimidinische Endonukleaseaktivität in each synthesis cycle n-Iten can by processing 5 ' - phosphates for the next reaction sequence place will. Order all steps repeat themselves k-times to the latter ssDNA< RTI ID=14.34> Molekül< /RTI> in m-ten step one inserted Figure < RTI ID=15.1> 5~ " in vitro " - ssDNA synthesis in S'-3' direction (1) < /RTI> To one < RTI ID=15.2> Matrix coupled starter molecule (n) is einer< by means of; /RTI> < RTI ID=15.3> Ligaseaktivität um< /RTI> a n+Ites < RTI ID=15.4> Einzelstrangmolekül< /RTI> by a 3 ' < RTI ID=15.5> S'Phosphodiesterbindung link-linking those n-FIte ssDNA besitzt< /RTI> terminal < RTI ID=15.6> Uracildesoxynukleotid, < /RTI> is < RTI ID=15.7> 5 ' - phosphoryliert< /RTI> and 3 ' - blocked < RTI ID=15.8> (- X) - Die< /RTI> glycosidische connection of the cousin uracil is < by; RTI ID=15.9> DNA Uracilglycosylase split, < /RTI> whereby a apyrimidinische position results these again from a apyrimidinische is < RTI ID=15.10> Endonukleaseaktivität (ExonukleaseIII) < /RTI> so split that < RTI ID=15.11> 5 ' - Phosphat< /RTI> and a 3 ' - OH ends to developing (3) to < RTI ID=15.12> freiwerdende< /RTI> 5 ' - phosphate end is < in the n+2ten Ligationsreaktion; RTI ID=15.13> n-F2te< /RTI> < RTI ID=15.14> SsDNA Molekülc /RTI> < RTI ID=15.15> links Eine< /RTI> anschliet3ende terminal Transferasereaktion with one < RTI ID=15.16> Didesoxytrinukleotid (nicht< /RTI> shown, 5. Figure 7.) inactivates all DNA chains for the n+3ten Ligationsschritt for all following Ligationsschritte, if none < RTI ID=15.17> n+2tes ssDNA molecule im< /RTI> n+2ten step inserted become through < RTI ID=15.18> DNA Uracilglycosylase< /RTI> and the apyrimidinische Endonukleaseaktivität can by processing 3 ' - OH for < RTI ID=15.19> nächste< /RTI> Reaction sequence (n+3) to < RTI ID=15.20> Verfugung< /RTI> are placed. All steps repeat themselves k-times to the last ssDNA molecule in m-ten step inserted wurde< RTI ID=15.21> ?Invitro? - ssDNA-Synthesein5'-3' direction (1) EinanFigur6. starter-molecule-coupled (n) becomes by means of one Ligaseaktivitat uln a n+Ites single strand molecule by a 3 ' 5 Phosphodiesterbindung verknüpft A112 further Schritte< /RTI> take place as < in figure 5 represented; RTI ID=15.22> Irn< /RTI> last step knows, < RTI ID=15.23> initiiert< /RTI> by for example one < RTI ID=15.24> 3 ' - < /RTI> terminal < RTI ID=15.25> Haarnadelstruktur< /RTI> a 3 ' - end < RTI ID=15.26> for one DNA < /RTI> < RTI ID=15.27> Polymerisationsreaktion< /RTI> to < RTI ID=15.28> Verfügung< /RTI> placed or one dsDNA polymerisation taken place as in figure 2 are beschrieben< RTI ID=15.29>
Figure 7. ?In vitro? - ssDNA synthesis in 5 ' - 3 ' - direction (1),
Display of the reaction to the inactivation not more ligierter
Enden-Eine terminal Transferasereaktion with einem< /RTI> < RTI ID=15.30> Didesoxytrinukleotid< /RTI> inactivates all DNA chains for < RTI ID=15.31> nächsten< /RTI> Ligationsschritt for all following in each case Ligationsschritte, if none < RTI ID=15.32> SsDfA Molekül< /RTI> in n-Iten
Synthesis step inserted was < by; RTI ID=15.33> DNA Uracilglycosylase< /RTI> and the apyrimidinische < RTI ID=15.34> Endonukleaseaktivität< /RTI> can through
Processing 3 ' - OH for < RTI ID=15.35> nächste< /RTI> Reaction sequence made available all steps repeat themselves k times to the latter < RTI ID=15.36> SsDNA molecule im< /RTI> m-ten step one inserted
Figure 8. Was based DNA synthesis. < RTI ID=15.37> Startermolekül< /RTI> (n) to one < RTI ID=15.38> Verfügung< /RTI> placed.

Synthetic Oligonukleotide is < RTI ID=15.39> sequentiell< /RTI> in a cyclic reaction sequence to < RTI ID=15.40> Verfügung< /RTI> placed, whereby of them < RTI ID=15.41> 5 ' - Ende< /RTI> for hybridizing with preceding in each case < RTI ID=15.42> 3 ' - Ende< /RTI> the complementary DNA strand one brings. Of < RTI ID=15.43> 3 ' - Ende< /RTI> outgoing the synthesis takes place to
Doubling rank. Fig. 8A shows one < RTI ID=15.44> vollständige< /RTI> Degradation of the Template molecule with T7 (Gen6), during in Fig. 8B a partial degradation with Exonuklease III is represented.

The following examples serve the explanation of the present invention.

Example 1 the recursive < RTI ID=16.1> DNA synthesis " in< /RTI> vitro " can for manipulation of < RTI ID=16.2> DNA sequences " in< /RTI> vitro " to be used. On the one hand Genmutationen can, as < RTI ID=16.3> Deletionsmutagenesen, < /RTI> also several Deletionen in a gene simultaneous, gene fusions under production of new properties, Insertionsmutagenesen, < RTI ID=16.4> Substitutionmutagenesen< /RTI> and also sequence inversions < RTI ID=16.5> durchgeführt< /RTI> become. The moreover can one be introduced to as many as desired point mutations into a sequence. All DNA sequences can without Zwischenclonierungsschritte in < RTI ID=16.6> parallelen< /RTI> Syntheses to be produced directly.

The functional changes of the biological resulting by the sequence manipulations < RTI ID=16.7> Activity " in vivo " können< /RTI> affect to the one the protein level, if the coding sequences into functional proteins < RTI ID=16.8> translatiert< /RTI> will can. The method can thus for the conversion of considerations with Enzym-bzw. Protein Design to be used.

On the other hand however it is possible to manipulate the DNA sequences of regulatorischer cis elements around those

< RTI ID=16.9> Bindungsaktivität< /RTI> from Transaktivatoren and Suppresoren to to change to examine of them behavior or completely new combinations of < RTI ID=16.10> Cis Elementen< /RTI> to create. Further < RTI ID=16.11> könnte< /RTI> also < RTI ID=16.12> Aktivität< /RTI> of < RTI ID=16.13> RNA Molekülen< /RTI> are manipulated (z. B. Ribozyme), if the manipulated DNA is transliterated.

The following example of the use of the recursive DNA ?in vitro? - synthesis method is those Manipulation from DNA sequences to the analysis < RTI ID=16.14> Bindungsaktivität< /RTI> a transaktiven Modulator protein at one < RTI ID=16.15> bakteriellen< /RTI> Activator region. Through < RTI ID=16.16> DIX, in< /RTI> vitro " - Mutagenese < RTI Kompetitors (i. D. R. < RTI ID=17.1> polydIdC), < /RTI> from the activator range, this points < to the separation of the components, RTI ID=17.2> Inkubationsansatzes< /RTI> in the electrical field of a native PAA gel a clear retention of the DNA fragment opposite one < RTI ID=17.3> Kontrollansatz< /RTI> without addition of raw excerpt protein.

To the identification < RTI ID=17.4> NicR1-Bindeaktivität< /RTI> consulted experimental beginning is called gel retention analysis. With < RTI ID=17.5> Hilfe< /RTI> this method can the kinetic and functional behavior of DNA Bindungsproteinen in < RTI ID=17.6> Abhängigkeit< /RTI> from different < RTI ID=17.7> Parameters " in< /RTI> vitro " qualitatively and quantitatively to be examined. < RTI ID=17.8> Ausserdem< /RTI> one can make under certain conditions also statements the structure of the DNA/Protein complex.

Using raw excerpts one can usually recognize a dominant factor, retardierte gang in the gel retention experiment. A second gang is sometimes likewise recognizable. This DNA< RTI ID=17.9> Bindungsaktivität< /RTI> if nonspecific Kompetitor DNA could not be supprimiert by large quantities of more unmarkierter, < RTI ID=17.10> wohl< /RTI> but by unmarkiertes binding fragment in very small quantities. It was therefore accepted that this < RTI ID=17.11> DNA Bindeaktivität< /RTI> is specific and with the transkriptionellen
Regularization of the Nikotinregulons in connection stands. It was marked with the contraction NicR1 (nicotine modulator 1) (Mauch et < RTI ID=17.12> aluminium, < /RTI> Bernauer et < RTI ID=17.13> aluminium, < /RTI> 1992).

The behavior of the NIC g 1 napkin activity in < RTI ID=17.14> Gelretentionsexperiment< /RTI> in this work analyzed over
Statements < RTI ID=17.15> über< /RTI> the place, which < specificity, kinetics and; RTI ID=17.16> Stöchiometrie< /RTI> to be able to meet the connection reaction and examine the reaction of the DNA Bindungsfunktion on manipulations at the WT-6-HDNO-Promotorfragment and for potential Effektorsubstanzen.

These attempts should < RTI ID=17.17> darüber< /RTI> < RTI ID=17.18> Aufschluss< /RTI> give, which molecular mechanisms for the regularization of the 6-HDNO-Gens be responsible could. < RTI ID=17.19> Ausserdem< /RTI> the rearing conditions were < and; RTI ID=17.20> Induktionsstatus< /RTI> the Arthrobacter < RTI ID=17.21> nicotinovorans< /RTI> , From those raw excerpt finally varies cells to
Analysis in < RTI ID=17.22> Gel retention experiment hergestellt< /RTI> became. These experiments should < RTI ID=17.23> Aufschluss< /RTI> < RTI ID=17.24> < /RTI> give whether < itself; RTI ID=17.25> Bindungsverhalten< /RTI> of < RTI ID=17.26> NicR1< /RTI> changed or the presence < RTI ID=17.27> zusätzlicher< /RTI> Factors in < RTI ID=17.28> Abhängigkeit< /RTI> from one the attempt parameter to prove is. To the Protein/DNA
Connection attempts used < RTI ID=17.29> Reaction standard buffer lehnt< /RTI> itself to the reaction buffer used by Garner and Revzin (1981) on. < RTI ID=17.30> NicR1-Bindungsaktivität< /RTI> cracking is enrichable by ammonium sulphate. The enrichment < RTI ID=17.31> NicR1-Bindungsaktivität< /RTI> was the condition for
Attempts to the analysis < RTI ID=17.32> Bindungsverhaltens< /RTI> of < RTI ID=17.33> NicR1< /RTI> when simultaneous connection of both palindromischen sequences, which are to be found on the WT-6-HDNO-Promotorfragment.

The WT-6-HDNO-Promotorfragment from that 5 ' - controlled area of the 6-HDNO-Gens possesses some very interesting < RTI ID=17.34> Sequenzmerkmale< /RTI> (S. Fig. 7). It is from expanded inverted repetitions of sequence (IR) and other remarkable motives for sequence < RTI ID=17.35> geprägt.< /RTI> Characteristic sequence arrangements within the 6-HDNO-Gen-promoterregion are in Fig. 7 shown. This shows two inverted
Repetitions, < RTI ID=17.36> IR1< /RTI> and < RTI ID=17.37> IR2, < /RTI> which extensive Homologien among themselves have (Fig. 7). The right palindromische half side of IR2 repeats itself within 5 ' - the range again. Such Palindrome is structural features, which one finds in many bacterial cis active modulator regions.

< RTI ID=18.1> IR1< /RTI> and < RTI ID=18.2> IR2< /RTI> 50 BP are separated a long interpalindromische sequence by. The palindromischen half sides of IR1 are < RTI ID=18.3> über< /RTI> 17 BP to each other homologous, of IR2 < RTI ID=18.4> über< /RTI> 9 BP. The Palindrom of IR1 achieves an expansion larger around twelve pairs of cousins, shows however in this range two Insertionen of ever two and a pair of cousins (RK, A). Ten of twelve pairs of cousins of IR1 in < RTI ID=18.5> 5 ' - Hälfte< /RTI> and 9 of 12 pairs of cousins in < RTI ID=18.6> 3 ' - Hälfte< /RTI> the sequence are homologous to IR2 (Fig. 7A, sequences of IR1 and IR2). These sequence-specific features would know structural and functional meaning with the connection destin trans " - connection-active protein < RTI ID=18.7> NicR1< /RTI> and one < RTI ID=18.8> S70-ähnlichen< /RTI> < RTI ID=18.9> RNA Polymerase< /RTI> possess. IR1 < RTI ID=18.10> repräsentiert< /RTI> one almost perfect < RTI ID=18.11> S70-ähnliche< /RTI> Activator sequence, with a remarkable modification. The 10-region differs from the Konsensussequenz ACT AAT by the Insertion of a C, whereby the sequence ACT CAAT develops. In the sequence of IR2 one finds eine-30-Region, but none < RTI ID=18.12> Ähnlichkeit< /RTI> to the well-known 10-region one < RTI ID=18.13> S70-ähnlichen< /RTI>
Activator sequence. The spacing der-10 und-30-Region corresponds to the S70-Ideal of 17 with 16 BP.

Integrated into the sequence of IR2 die-35-Region one is < RTI ID=18.14> S70-ähnlichen< /RTI> Promoter, one < RTI ID=18.15> konsensusähnliche-10-Region fehlt.< /RTI> Some different < RTI ID=18.16> Sequenzmerkmale< /RTI> could also < RTI ID=18.17> Aktivität< /RTI> further to < RTI ID=18.18> 5 ' - Sequenzbereich< /RTI> the 6-HDNO-Gens of transaktiver regulatorischer elements reflect.

< RTI ID=18.19> Innerhalb< /RTI> the Palindrome IR1 and IR2 are three Nlal (CATG) - Erkennungspalindrome at homologous position. < RTI ID=18.20> Interessant< /RTI> it is that behind the left palindromischen half side of <

itself; RTI ID=18.21> IR2, < /RTI> at not homologous position, likewise such an interface < RTI ID=18.22> befindet.< /RTI> The question of coincidence or necessity of one arises < RTI ID=18.23> solchen< /RTI> Structure. Outside of the palindromischen sequences of IR1 and < RTI ID=18.24> IR2< /RTI> are likewise < RTI ID=18.25> auffallende< /RTI> Motives for sequence. GC-und RK realms sequences are alternating arranged. An interesting sequence characteristic of this domain is the presence of office enriches
Sequence sections, which are interrupted by RK realms a sequence section in the 6-HDNO-5' sequence. The office sequence blocks are < above; RTI ID=18.26> 5 ' - Region< /RTI> < RTI ID=18.27> 570-ähnlichen< /RTI> Promoter locates. A detailed < RTI ID=18.28> Basennachbarschaftsanalyse< /RTI> to the algorithm described in ebbing oils and Zalkin (1989) points, < RTI ID=18.29> dass< /RTI> this sequence in high < RTI ID=18.30> Masse< /RTI> not statistical is. In order to show this, particularly a computer program was < RTI ID=18.31> in " Pascal " written. Während< /RTI> the sequences within the palindromischen ranges out quite < RTI ID=18.32> regelmässig< /RTI> alternating short GC-und RK
Ranges with very balanced office content, are 5 ' to the Palindromen larger exist
Sequence sections with very unbalanced office content (Fig. 7A). < RTI ID=18.33> Zunächst< /RTI> the office content of < rises; RTI ID=18.34> 5 ' ausserhalb< /RTI> the Palindroms < RTI ID=18.35> IR2< /RTI> coming on, it becomes first an office maximum, then office
Go through minimum (Fig. 7A). The situation repeats itself before < RTI ID=18.36> Palindrom< /RTI> IR1. < RTI ID=18.37> Alternierende< /RTI> Office and RK realms of sequence sections become with structural properties of the protein connection in
Connection brought. RK realms the positions turn with their small DNA furrow into that
Protein, which point office realms sequence blocks to < RTI ID=18.38> aussen.< /RTI> Office realms promoters, which < with the well-known; RTI ID=18.39> S70-ähnlichen< /RTI> Promoters no more sequence similarity possess, are in Streptomyces species. As starter molecule (S. < RTI ID=19.1> Fig. 7M (0)) für< /RTI> the recursive DNA synthesis was < RTI ID=19.2> Plasmid< /RTI> pUC19 (Yanish Perron et. < RTI ID=19.3> aluminium, < /RTI> 1985) with < RTI ID=19.4> BamHi< /RTI> and KpnI doubledigests and < RTI ID=19.5> über< /RTI> a agar eye gel cleaned. KpnI has the recognition sequence 5 ' - GGTAC'C-3'. To < RTI ID=19.6> 3 ' - überstehende< /RTI> < RTI ID=19.7> KpnI Ende< /RTI> one < RTI ID=19.8> Oligonukleotid< /RTI> complementary sequence in presence of a T4-Ligase, T4-DNA-Polymerase and 0.2 mm < RTI ID=19.9> dNTP< /RTI> under standard conditions (Sambrook et < RTI ID=19.10> aluminium, < /RTI> 1989)) angelagert, ligiert and to doubling rank < RTI ID=19.11> aufgefüllt. < /RTI> The Oligonucleotid possesses to 5 ' - end the recognition sequence < RTI ID=19.12> Restriktionsendonuclease< /RTI> < RTI ID=19.13> RleAI plus< /RTI> some < RTI ID=19.14> zusätzliche< /RTI> Bases (S. Fig. 7A < RTI ID=19.15> (1)). < /RTI> Now < RTI ID=19.16> doppelsträngige DNA molecule (aufgefülltes< /RTI> < RTI ID=19.17> überstehendes< /RTI> synthetic Oligonucleotid) was < with an enrichment parliamentary group; RTI ID=19.18> Restriktionsendonuclease< /RTI> RleAI from Rhizobium leguminosarum restringiert (in each case Fig. 7 (2) and (3 ')). The reaction conditions were et < from Veseley; RTI ID=19.19> aluminium, < /RTI> (1990) taken. This enzyme produces 3 ' supernatant ends outside of its asymmetric connection place. This < RTI ID=19.20> Spezifität< /RTI> is so far singular and < RTI ID=19.21> left; /RTI> the repeated accumulation of a Oligonucleotides and the Priming for a DNA < RTI ID=19.22> Polymerisation< /RTI> too. The short DNA fragment with the RleAI Erkennungssequenz was < of; RTI ID=19.23> Plasmid< /RTI> < RTI ID=19.24> über< /RTI> < RTI ID=19.25> Agarosegel< /RTI> away-cleaned. To with the restriction reaction the developing < RTI ID=19.26> 3 ' - überstehende< /RTI> End became again a Oligonucleotid complementary to its end (Fig. 7A (2)) angelagert and < RTI ID=19.27> filled up, < /RTI> as mentions above (S. also Fig. 1 and 2). The same reaction became with the Oligonucleotid (Fig. 7A (3)) and the variants Fig. 7B-1 to B-7 < RTI ID=19.28> durchgeführt.< /RTI> By the use of synthetic
Oligonucleotiden knew parallel seven sequence variants and < RTI ID=19.29> Wildtypsequenz< /RTI> are produced.

After the reaction sequence Fig. 7A (3) were < newly developed the DNAs with; RTI ID=19.30> BamHI nachgespalten< /RTI> (S.

Sequence, Fig. 7A (3 ")), the vector (pUC19 + binding fragment) zirkularisiert and in accordance with standard methods in E. coli transforms. If the RleAI Erkennungssequenz terminal at the end also the synthetic Oligonucleotide is, one knows the DNA Synthesereaktion as < in this example in each case around a step; RTI ID=19.31> verlängern.< /RTI>

Around the connection characteristics of < RTI ID=19.32> NicR1< /RTI> to characterize, changes of sequence were < into those; RTI ID=19.33> S70~ähnlichen< /RTI> Promoter basic IR1-Bindungsstelle imported and the length the interpalindromische
Sequence < RTI ID=19.34> (IS, < /RTI> Fig. 7B-5, - 6, - 7) was varied. By letzere attempts the sterischen should Requirements to the palindromische connection sequence IR1 to be examined. Sequence modifications, which < into the WT-6-HDNO-Promotorfragment; RTI ID=19.35> eingeführt< /RTI> became, are shown in the figure 7.

< RTI ID=19.36> Changes, < /RTI> < RTI ID=19.37> through " rekursive< /RTI> DNA synthesis in vitro " in the sequence promoter of the containing < RTI ID=19.38> IR1-Palindroms< /RTI> and in the interpalindromischen sequence < RTI ID=19.39> eingeführt< /RTI> became, are in Fig. 7B < RTI ID=19.40> dargestellt.< /RTI>

The reducing of < RTI ID=19.41> IR1< /RTI> on a Oktamer (Fig. 7B-3) like also the Deletion of the central G-position (Fig.

7B-4) destroy < RTI ID=19.42> Bindungsfähigkeit< /RTI> of < RTI ID=19.43> NicR1< /RTI> < on; RTI ID=19.44> IR1.< /RTI>

One sets < RTI ID=19.45> Spaltungsprodukte< /RTI> in < RTI ID=19.46> Gelretentionsexperiment< /RTI> , then only IR2 is retardiert, not however the mutated IR1 containing fragment. In Fig. 7B-4 shown Konstrukt shows retention only by the connection of < RTI ID=19.47> NicR1< /RTI> < on; RTI ID=19.48> IR2.< /RTI> Since the value of the complex at IR2 the same value has as that
< complex on; RTI ID=19.49> IR1, < /RTI> this is a reference to the connection of the same protein to both Palindrome.

< RTI ID=20.1> @< /RTI> Against < RTI ID=20.2> ausgeprägten< /RTI> Effect, which < by the changes; RTI

ID=20.3> sowohl< /RTI> the length like also the symmetry of the Palindroms IR1 on < RTI ID=20.4> NicR1-Bindung< /RTI> one produces, led changes of the number of Helixwindungen in the interpalindromischen sequence to no difference < RTI ID=20.5> NicR1-Bindung< /RTI> to both Palindrome. The length < RTI ID=20.6> interpalindromischen< /RTI> Sequence became by Deletionen like also Insertionen of for each 5bp (Fig. 7B-6 und-7) < RTI ID=20.7> verändert.< /RTI> These changes correspond to one half Helixwindung each. As consequence it results from the fact that in these DNA mutants < itself; RTI ID=20.8> IR2-< /RTI> Connection place relative to < RTI ID=20.9> IR1-Bindungsstelle< /RTI> over < RTI ID=20.10> 180 < /RTI> rotated finds. Additionally the 50 BP long interpalindromische sequence was reduced < around 20 BP; RTI ID=20.11> (Fig.< /RTI> 7B-5). The pattern < RTI ID=20.12> Gel retention experiment, which trägt< these changes; /RTI> (Fig. 7B-5, - 6, - 7), was identical to the control sample, which < with; RTI ID=20.13> unveränderten< /RTI> 242 BP are enough for 6-HDNO-Promotorfragment < RTI ID=20.14> (Fig.< /RTI> 7B-1) to see was.

The right stops of IR1 die-10 region of the promoter of the 6-HDNO-Gens contains of the Konsensussequenz of the promoter < RTI ID=20.15> S70~RNA-Polymerasen< /RTI> by the Insertion cytosine of a containing, < RTI ID=20.16> zusätzlichen< /RTI> Cousin position in < RTI ID=20.17> TATAAT Sequenz< /RTI> differentiates (Fig. 7B-1). The question arose whether this unusual < RTI ID=20.18> S7 -10-Region< /RTI> to < RTI ID=20.19> Spezifität< /RTI> < RTI ID=20.20> NicR1-Bindung< /RTI> at IR1 portion has. < RTI ID=20.21> Im< /RTI> < RTI ID=20.22> Gelretentionsexperiment< /RTI> (Fig. 7B-2) with < RTI ID=20.23> NicR1< /RTI> the Deletion of the cytosine remainder at the appropriate position (Fig showed. 7B-2) no change of the protein connection sample, compared to that Pattern, which < with; RTI ID=20.24> unveränderten< /RTI> DNA fragment received was < RTI ID=20.25> wohl< /RTI> but < with the connection; RTI ID=20.26> S 70< /RTI> < RTI ID=20.27> - ähnlichen< /RTI> RNA polymerase of E. coli. < RTI ID=20.28>

The statement that the two mutations Fig. 7B-3 und-4 the NIC g 1 napkin ability to the Palindrom< /RTI> IR1 strongly reduce, if prevent not even completely, becomes by further, here not described Attempts supports.

Example 2
Example of the synthesis of the: < RTI ID=20.29> PLASMIDS n-AN7 885 BP: Huang, Little, Seed (1985) in: < /RTI> Vectors: A molecularcloningandtheirapplications ", OF
Rodriguez, R., OD., Stoneham, Publishers, mA, the USA.

STARTER MOLECULE: < RTI ID=21.1>
AAUGCGGCCGCTCACGAGGCCGCCGGTTAATTAACTCGAGAABTCCGCGGTGCAATTAATT x of restriction enzymes: EagI, Bst2BI, AccBSI, NotI, PacI, XhoI, EcoRI, SacI< /RTI> B=Biotin X=AminoBlock < RTI ID=21.2> JE-AN7-SEQUENCE: SOURCE: GeneBank< /RTI> POSITION 3: Uracil < RTI ID=21.3> 01
AAUTTTCGGACTTTTGAAAGTGATGGTGGTGGGGGAAGGATTCGAACCTTCGAAGTCGATGAc3'& lt; /RTI> 02 < RTI ID=21.4>
AAUGGCAGATTTAGAGTCTGCTCCCTTTGGCCGCTCGGGAACCCCACCACGGGTAATGCTTTT3'& lt; /RTI> 03 < RTI ID=21.5>
AAUACTGGCCTGCTCCCTTATCGGGAAGCGGGGCGCATCATATCAAATGACGCGCCGCTGTAA3'& lt; /RTI> < RTI ID=21.6> 04
AAURGTGTTACGTTGASAAAGAATTCCCGGGGATCCGTCGACCTGCAGATCTCTAGAAGCT'E-3'& lt; /RTI> 05 < RTI ID=21.7>
AAUCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT-3'& lt; /RTI> 06 < RTI ID=21.8>
AAUCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAA-3'& lt; /RTI>
07 < RTI ID=21.9> AAUGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTC-3'& lt; /RTI>
< RTI ID=21.10>
08 AAUTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGT-3'& lt; /RTI>
09 < RTI ID=21.11> AAUAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCG-3'&
lt; /RTI> < RTI ID=21.12>
010 AAUCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGG-3'
011 AAUCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT-3' @< /RTI>
012 < RTI ID=21.13> AAUTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGC-3'&
lt; /RTI>
013 AAUTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG-3'
014 AAUCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTC-3'
015 < RTI ID=21.14> AAUAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAAATTC-3'& lt; /RTI> < RTI ID=22.1>
LAST SYNTHESEZYKL US: B=Biotin-UCTCGAGAATTCCGCGGTGCTTAATTAAAAAAAAAAAAA Underscore: SupF< /RTI> < RTI ID=22.2> BlueSequence< /RTI> : Polylinker < RTI ID=22.3> BlackSequence< /RTI> : < RTI ID=22.4> ColEI< /RTI> < RTI ID=22.5> Kurzprotokoll < /RTI> Ug a biotinyliertes starter molecule was < on; RTI ID=22.6> streptavidingecoatete< /RTI> DynalBeads bind. Then 0-2 mm one < RTI ID=22.7> Biotin desoxyUracil adjusted (ever 0 5 h Inkubation with blank), um< /RTI> to block all biotin connection places.

In 16 synthesis cycles (Ligation, T4-RNA-Ligase; Uracil DNA Glycosyllase, < RTI ID=22.8> ExonukleaseIII< /RTI> ; Phosphatase) became the 17 DNA< RTI ID=22.9> Molekule< /RTI> to one < RTI ID=22.10> einzelsträngigen, < /RTI> the whole Plasmidsequenz comprising DNA links.

Of the 3 ' - end starter molecule by means of the T4-DNA polymerase ssDNA to dsDNA was < ago; RTI ID=22.11> filled up with NotI< /RTI> dsDNA from your connection to the DynalBeads one released.

The same quantity of fresh DynalBeads was < RTI ID=22.12> zugegeben.< /RTI>

Only < RTI ID=22.13> Moleküle< /RTI> with biotin to the column were gebunden< RTI ID=22.14> Moleküle< /RTI> without biotin from the last Ligationsreaktion away-washed with the Restriktionsendonuklease PacI one after-split.

The Dynabeads was < with one; RTI ID=22.15> Magneten< /RTI> pelletiert.

From < RTI ID=22.16> Überstand< /RTI> were < RTI ID=22.17> Moleküle< /RTI> with ethanol < RTI ID=22.18> gefällt< /RTI> and < RTI ID=22.19> Molekül< /RTI> with < RTI ID=22.20> T4-DNA-Ligase< /RTI> < RTI ID=22.21> zirkularisiert ?< /RTI> The zirkularisierten synthetic Plasmidmoleküle was then < in; RTI ID=22.22> E. coli DH10?/P3< /RTI> transformed after < RTI ID=22.23> Standardprotokoll< /RTI> and against Tet/Amp aud LB-plates

selektioniert.

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